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# Arthritis is Developed in *Borrelia*-primed and -infected Mice Deficient of Interleukin-17

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Interleukin-17 (IL-17) has been shown to participate in the development of Lyme arthritis in experimental mice. For example, neutralization of IL-17 with antibodies inhibits induction of arthritis in *Borrelia*-primed and -infected C57BL/6 wild-type mice. We hypothesized that mice lacking IL-17 would fail to develop *Borrelia*-induced arthritis. IL-17-deficient and wild-type C57BL/6 mice were primed with

heat-inactivated *Borrelia* and then infected with viable spirochetes 3 weeks later. No swelling or major histopathological changes of the hind paws were detected in IL-17-deficient or wild-type mice that were primed with *Borrelia* or infected with viable spirochetes. By contrast, IL-17-deficient and wild-type mice that were primed and subsequently infected with heterologous *Borrelia* developed severe swelling and histopathological changes of the hind paws. In addition, *Borrelia*-primed and -infected IL-17-deficient mice exhibited elevated gamma-interferon (IFN- $\gamma$ ) levels in sera and increased frequencies of IFN- $\gamma$ -expressing lymphocytes in popliteal lymph nodes compared to *Borrelia*-primed and -infected wild-type mice. These results demonstrate that IL-17 is not required for development of severe pathology in response to infection with *Borrelia burgdorferi*, but may contribute to disease through an interaction with IFN- $\gamma$ .

**Keywords:** Lyme arthritis, *Borrelia burgdorferi*, interleukin-17, gamma-interferon, T cells

## Introduction

Lyme borreliosis, caused by *Borrelia burgdorferi sensu lato* spirochetes, is the most common tick-borne human disease reported in the USA and Europe. Arthritis is one of the most frequent late disease manifestations of Lyme borreliosis, affecting ~60% of infected, but untreated, individuals in North America (Steere, Schoen and Taylor 1987). Despite antimicrobial treatment, 10% of these arthritic patients develop chronic joint inflammation (Steere, Schoen and Taylor 1987), resulting in permanent cartilage and bone damage, causing pain and affecting mobility.

The immune mechanisms responsible for the development of Lyme arthritis are partially understood. We have provided evidence that interleukin-17 (IL-17), a pro-inflammatory cytokine produced by T helper (Th) 17 cells, is involved in the development of *Borrelia*-induced arthritis in a mouse model of disease (Burchill *et al.*2003; Nardelli *et al.*2004, 2008; Kotloski *et al.* 2008; Kuo *et al.*2011; Hansen *et al.*2013). IL-17 has also been shown to participate in the development of rheumatoid arthritis, collagen-induced arthritis and other chronic inflammatory diseases (Chabaud *et al.*2000; Lubberts *et al.*2004; Agarwal, Misra and Aggarwal 2008; Pinto *et al.*2010; Isailovic *et al.*2015; Owaga *et al.*2015). Importantly, Infante-Duarte *et al.* (2000) showed that IL-17 was produced by synovial T cells of human patients with Lyme arthritis in response to borrelial antigens. Subsequently, Codolo *et al.* (2008, 2013) showed that IL-17 was secreted by synovial T cells of human patients with Lyme arthritis after stimulation with neutrophil-activating protein A of *B. burgdorferi*. These findings, along with our previous work describing the involvement of Th17-associated cytokines (Burchill *et al.*2003; Nardelli *et al.*2004, 2008; Kotloski *et al.*2008; Kuo *et al.*2011; Hansen *et al.*2013), suggest that IL-17 is involved in the pathogenesis of arthritis following infection with *B. burgdorferi*.

We previously used a model with *Borrelia*-primed and -infected wild-type and gamma interferon (IFN- $\gamma$ )-deficient C57BL/6 mice for inducing arthritis (Burchill *et al.*2003; Christopherson *et al.*2003; Nardelli *et al.*2004, 2005, 2006, 2008; Amlong *et al.*2006; Kotloski *et al.*2008; Kuo *et al.*2011; Hansen *et al.*2013) that is driven by T cells (Lim *et al.*1995a,b), as occurs in humans with later-stage Lyme disease (Chen *et al.*1999; Pianta *et al.*2015). We showed that mice primed with heat-inactivated *Borrelia* and subsequently infected with a live, heterologous borrelial strain developed severe, destructive, IL-17-dependent arthritis in the absence of IFN- $\gamma$  (Burchill *et al.*2003; Nardelli *et al.*2004). In addition, we showed (Nardelli *et al.*2006, 2008; Kotloski *et al.*2008; Kuo *et al.*2011) that IL-17-dependent arthritis of moderate severity also developed in *Borrelia*-primed and -infected wild-type mice. Infiltration of lymphocytes was observed in the synovial and subsynovial tissues of *Borrelia*-primed and -infected mice compared to control groups (Kotloski *et al.*2008). Furthermore, sera and lymph node cell cultures from arthritic *Borrelia*-primed and -infected mice showed increased levels of IL-17 compared to control groups (Amlong *et al.*2006; Kotloski *et al.*2008; Kuo *et al.*2011). Neutralization of IL-17 inhibited the development of arthritis in these mice (Burchill *et al.*2003; Nardelli *et al.*2004, 2005, 2008). This demonstrates that IL-17 and, possibly, T lymphocytes, such as Th17 cells, play a role in the induction of arthritis in the *Borrelia*-primed and -infected murine model of Lyme arthritis.

Although there is considerable evidence that IL-17 is associated with the development of Lyme arthritis (Codolo *et al.*2008, 2013; D'Elia, Codolo and de Bernard 2009; Oosting *et al.*2011), in addition to our work with *Borrelia*-primed and -infected mice (Burchill *et al.*2003; Christopherson *et al.*2003; Nardelli *et al.*2004, 2005, 2006, 2008; Amlong *et al.*2006; Kotloski *et al.*2008; Kuo *et al.*2011; Hansen *et al.*2013), we questioned whether IL-17 is required for the induction of Lyme arthritis. In this study, we hypothesized that arthritis would not develop in IL-17-deficient *Borrelia*-primed and -infected C57BL/6 mice. However, IL-17-deficient *Borrelia*-primed and -infected C57BL/6 mice developed arthritis, indicating that IL-17 is not solely responsible for the pathogenesis of arthritis in this animal model.

## Materials and Methods

### Mice

Six to eight week-old male IL-17-deficient C57BL/6 mice were provided by Dr Bruce Klein and Dr Marcel Wuethrich (University of Wisconsin School of Medicine and Public

Health) with permission from Dr Yoichiro Iwakura (University of Tokyo's Institute of Medical Science). Wild-type mice of the same age were purchased from Jackson Laboratory (West Sacramento, CA, USA). All mice were kept at the animal facility located at the University of Wisconsin School of Medicine and Public Health. Mice were maintained under a pathogen-free condition at 21°C with food and acidified water provided *ad libitum* during daily cycles of 12 h of light and darkness. Experimental protocols were approved by the Animal Care and Use Committee of the University of Wisconsin School of Medicine and Public Health.

## Organisms and preparation

Low-passage-number (<10) virulent *Borrelia bissettii* and *B. burgdorferi* isolate 297 were cultured in modified Barbour-Stoenner-Kelly (BSK) medium until reaching a concentration of  $10^7$  spirochetes/ml. Samples of 500  $\mu$ l were dispensed into 1.5 ml screw cap tubes (Sarstedt; Newton, NC, USA) containing 500  $\mu$ l of BSK medium with 10% glycerol (Sigma-Aldrich; St. Louis, MO, USA). Spirochetes were stored at -70°C until used.

## Priming of mice

A frozen suspension of *B. bissettii* was thawed, heated in a water bath at 56°C for 30 min and examined by dark field microscopy. No motility was detected. In addition, an aliquot of the heat-inactivated suspension of *B. bissettii* failed to grow in fresh BSK medium after 7 days of incubation. Heat-inactivated *B. bissettii* organisms were then washed three times by centrifugation at  $10\ 000\times g$  with ice-cold phosphate-buffered saline (PBS, pH 7.4) that was filter-sterilized with a 0.2- $\mu$ m-pore-size filter (Gelman Sciences; Ann Arbor, MI, USA). Subsequently, washed *B. bissettii* organisms were resuspended in PBS and mixed with a volume of 1% aluminum hydroxide (Reheis; Berkeley Heights, NJ, USA) to yield a concentration of  $2 \times 10^6$  spirochetes/ml. Mice were then anesthetized with isoflurane using a nose-and-mouth cup and injected subcutaneously in the sural region near the popliteal lymph node of the hind limb with 0.10 ml of the vaccine using a 23-gauge needle.

## Infection of mice

A frozen suspension of *B. burgdorferi* isolate 297 was thawed and mixed with 4 ml of fresh BSK medium. The spirochetal culture was incubated at 32°C until reaching a concentration of  $2 \times 10^7$  viable spirochetes/ml. Twenty-one days after priming of mice with heat-inactivated *B. bissettii*, mice were anesthetized with isoflurane using a nose-and-mouth cup and injected subcutaneously in the right hind paw with  $1 \times 10^6$  (50  $\mu$ l) *B.*

*burgdorferi* isolate 297 organisms with a 27-gauge needle. The heterologous infection with *B. burgdorferi* isolate 297 induces arthritis and is not affected by the presence of priming-induced anti-*B. bissettii* antibodies. We have shown that infection with viable heterologous *Borrelia* organisms can induce arthritis (Lim *et al.* 1995a,b). If priming and infection are conducted with the same isolate of *B. burgdorferi*, arthritis will not develop if specific borreliacidal antibodies are present (Croke *et al.* 2000). Controls also included mice injected with BSK medium.

## **Assessment of swelling**

Swelling of the right hind paws of mice was measured with a digital caliper (Mitutoyo American Corporation; Aurora, IL, USA) with a sensitivity of 0.01 mm prior to infection with *B. burgdorferi* isolate 297 and every other day for 8 days. Mice were anesthetized with isoflurane contained in a nose-and-mouth cup, and the width and thickness of each hind paw at the tibiotarsal joint were measured. Caliper values within a group were averaged to obtain the mean caliper value for comparison of the degree of swelling.

## **Tissue preparation for histological examination**

Eight days after infection, mice were euthanized with isoflurane, and the right hind paw of each mouse was amputated at mid-femur. Paws were first placed in decalcifying solution (Lerner Laboratories; Pittsburgh, PA, USA) for 24 h, and then stored in 10% neutral buffered formalin solution for 48 h (Sigma-Aldrich). Paws were then placed in tissue-embedding cassettes (Fisher Scientific; Hanover Park, IL, USA), embedded in paraffin and cut into three to five 6- $\mu$ m-thick sections. Each section of the hind paws was placed onto a glass slide and stained with hematoxylin and eosin. Sections were cryptically coded, and a board-certified pathologist (T. F. Warner) performed fully blinded histopathological examinations of the tissues. The following ratings were used: 0, no histopathological changes; 1, mild infiltration of polymorphonuclear cells; 2, hyperplasia or villus formation with moderate infiltration of polymorphonuclear cells and lymphocytes; 3, severe hyperplasia with villus formation and infiltration of polymorphonuclear cells and lymphocytes.

## **Enzyme-linked immunosorbent assay**

Serum from each mouse was collected at day 8 after infection. IFN- $\gamma$  and IL-17 levels in each serum sample were measured with a Mouse Gamma-Interferon ELISA Ready-Set-

Go! Kit and Mouse Interleukin-17 ELISA Ready-Set-Go! Kit (eBioscience; San Diego, CA, USA), respectively. The change in optical density measured in 450 and 570 nm was obtained for each sample. A standard curve was generated, and data were expressed in picograms of IFN- $\gamma$  and IL-17 per milliliter of serum.

## Flow cytometry

Spleens and popliteal lymph nodes of mice were obtained 8 days after infection. Single-cell suspensions of lymph node cells and splenocytes were prepared by passing cells through a sterile nylon mesh screen (Fisher; Hanover Park, IL, USA) into cold filter-sterilized PBS in centrifuge tubes on ice. Red blood cells were lysed and discarded from suspensions of splenocytes. Total numbers of viable cells were determined using trypan blue exclusion and enumeration with a hemocytometer. The suspensions were diluted to  $1 \times 10^7$  cells/ml. A total of  $5 \times 10^5$  cells from each sample were then transferred to a sterile tissue culture treated 96-well plate (Corning; Corning, NY, USA). Cells were first incubated for 30 min on ice with Fixable Viability Dye eFluor 780 (eBiosciences) to differentiate viable from non-viable cells. Then, cells were incubated in the dark at 4°C for 30 min during surface staining with fluorescein isothiocyanate-conjugated rat anti-mouse CD4 antibody and Pacific Blue-conjugated rat anti-mouse CD8 antibody (BD Biosciences; San Jose, CA, USA). The antibody-to-cell ratio was determined to maximize recognition of CD4<sup>+</sup> or CD8<sup>+</sup> cells. Isotype controls were also included. After incubation, cells were permeabilized, and stained intracellularly with allophycocyanin-conjugated rat anti-mouse IFN- $\gamma$  antibody and phycoerythrin-conjugated rat anti-mouse IL-17 antibody (BD Biosciences) in the dark at 4°C for 30 min. Cells were then fixed with 5% formalin, and data were acquired using a BD LSRFortessa cell analyzer (BD Biosciences). Events were gated to include viable lymphocytes that were CD4<sup>+</sup> or CD8<sup>+</sup> and evaluated for expressing IFN- $\gamma$  or IL-17. The percentage of these specific cells is in relation to the entire lymphoid organ population. A total of 30 000 gated events were collected and analyzed with FlowJo single cell analysis software (Tree Star; Ashland, OR, USA).

## Statistics

Swelling of the hind paws among different groups of *Borrelia*-primed and -infected mice and concentrations of IL-17 were evaluated by Fisher's protected least-significant-difference test and one-way analysis of variance (ANOVA) through R Commander. The alpha level was set at 0.05. Data are presented as the average with two standard errors of the mean.

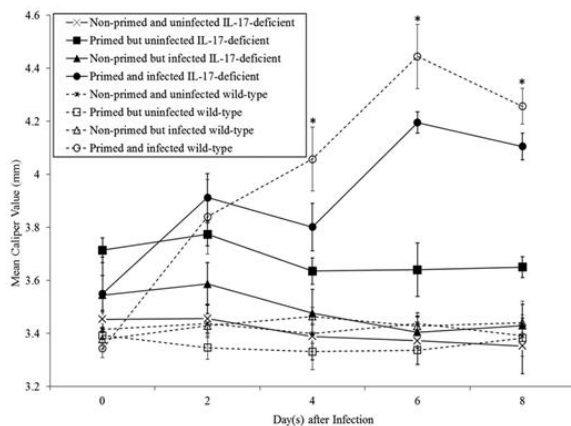
## Results

### Hind paw swelling in IL-17-deficient C57BL/6 mice

The purpose of this study was to determine if *Borrelia*-primed and -infected mice developed hind paw swelling in the absence of IL-17. Wild-type and IL-17-deficient mice were divided randomly into four groups: non-primed and uninfected but injected with BSK medium, *Borrelia*-primed but uninfected, non-primed but *Borrelia*-infected and *Borrelia*-primed and -infected. There were four mice in each group, and the study was replicated twice, using a total of 12 mice per group. Right hind paws of mice in each group were measured every other day for swelling. Mice were then euthanized on day 8 after infection, and their hind paws, sera, spleens and popliteal lymph nodes were harvested.

No swelling of the hind paws was detected in IL-17-deficient or wild-type C57BL/6 mice that were solely infected with *Borrelia burgdorferi* strain 297 or solely primed with *B. bissettii* (Fig. 1). By contrast, *Borrelia*-primed and -infected IL-17-deficient mice developed significantly ( $P < 0.05$ ) greater hind paw swelling between days 4 through 8 of infection than non-primed and uninfected, *Borrelia*-primed but uninfected or non-primed but *Borrelia*-infected mice of either genotype. However, the degree of swelling of *Borrelia*-primed and -infected wild-type mice between days 0 and 8 was notably greater than the overall swelling of *Borrelia*-primed and -infected IL-17-deficient mice. Similar results were obtained when this study was repeated twice.

Figure 1.



Development of hind paw swelling of IL-17-deficient and wild-type C57BL/6 mice that are non-primed and uninfected, *Borrelia*-primed but uninfected, non-primed but *Borrelia*-infected and *Borrelia*-primed and -infected. Error bars represent the mean hind paw swelling  $\pm$  two standard errors. Asterisks denote



a significant ( $P \leq 0.05$ ) difference in swelling among groups of mice. Data were obtained from three experiments. Twelve mice were used in total per group.

## Histopathological confirmation of arthritis

Tibiotarsal joints of *Borrelia*-primed and -infected IL-17-deficient and wild-type mice exhibited similar histopathological changes in the synovium and perisynovium (Table 1). Specifically, there was little difference between IL-17-deficient and wild-type mice in scores for synovial hyperplasia and villus formation, and infiltration of polymorphonuclear cells and lymphocytes into the perisynovium. By contrast, the histopathological changes in non-primed but *Borrelia*-infected IL-17-deficient mice were generally greater than the changes observed in non-primed but *Borrelia*-infected wild-type mice. Of these four groups of mice, non-primed but *Borrelia*-infected wild-type mice had the least pathology in the synovium. In the perisynovium, considerable lymphocytic infiltration occurred in *Borrelia*-primed and -infected IL-17-deficient and wild-type mice compared to non-primed but *Borrelia*-infected IL-17-deficient and wild-type mice. In addition, infiltration of neutrophils was significantly less in *Borrelia*-primed and -infected IL-17-deficient and wild-type mice compared to non-primed but *Borrelia*-infected IL-17-deficient and wild-type mice.

Table 1. Histopathological changes observed in the tibiotarsal joints of IL-17-deficient and wild-type C57BL/6 mice that are non-primed and uninfected, *Borrelia*-primed but uninfected, non-primed but *Borrelia*-infected and *Borrelia*-primed and -infected. Data were obtained from three experiments.

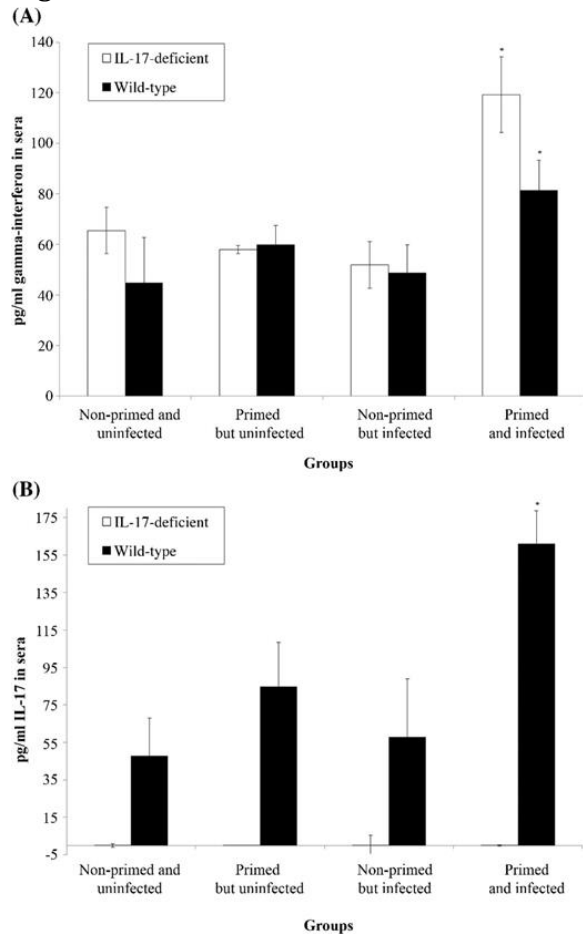
Group, with treatment and phenotype of mice	Synovium		Perisynovium	
	Hyperplasia Score	Villi formation Score	Polymorphonuclear cell infiltrations Score	Lymphocyte infiltrations Score
Non-primed and uninfected IL-17-deficient	0.00 <sup>a</sup>	0.00 <sup>a</sup>	0.00 <sup>a</sup>	0.00 <sup>a</sup>
Non-primed and uninfected wild-type	0.00 <sup>a</sup>	0.00 <sup>a</sup>	0.00 <sup>a</sup>	0.00 <sup>a</sup>
Primed but uninfected IL-17-deficient	0.00 <sup>a</sup>	0.00 <sup>a</sup>	0.00 <sup>a</sup>	0.00 <sup>a</sup>
Primed but uninfected wild-type	0.00 <sup>a</sup>	0.00 <sup>a</sup>	0.00 <sup>a</sup>	0.00 <sup>a</sup>
Non-primed but infected IL-17-deficient	1.15 <sup>b</sup>	0.15 <sup>a</sup>	2.13 <sup>c</sup>	1.91 <sup>c</sup>
Non-primed but infected wild-type	0.16 <sup>a</sup>	0.08 <sup>a</sup>	1.91 <sup>c</sup>	0.83 <sup>b</sup>
Primed and infected IL-17-deficient	1.25 <sup>b</sup>	0.50 <sup>b</sup>	0.83 <sup>b</sup>	2.42 <sup>d</sup>
Primed and infected wild-type	1.58 <sup>b</sup>	0.75 <sup>b</sup>	0.83 <sup>b</sup>	2.66 <sup>d</sup>

a–d Mean histopathological scores with different superscript in each category indicate significant difference from Fisher's protected least-significant-difference test, with alpha-value of 0.05 when comparing experimental groups with one another.

## IFN- $\gamma$ and IL-17 in sera and popliteal lymph node cells

We measured levels of IFN- $\gamma$  and IL-17 in sera to determine whether production of IFN- $\gamma$  and/or IL-17 was associated with the induction of arthritis in *Borrelia*-primed and -infected mice. Sera obtained from *Borrelia*-primed and -infected IL-17-deficient and wild-type mice had significantly greater levels of IFN- $\gamma$  than sera from non-primed and uninfected, *Borrelia*-primed but uninfected and non-primed but *Borrelia*-infected IL-17-deficient and wild-type mice (Fig. 2A). However, *Borrelia*-primed and -infected IL-17-deficient mice had significantly greater levels of IFN- $\gamma$  in sera compared to *Borrelia*-primed and -infected wild-type mice. As expected, no IL-17 was detected in sera of IL-17-deficient mice (Fig. 2B). Higher levels of IL-17 were detected in sera from arthritic *Borrelia*-primed and -infected wild-type mice compared to non-primed and uninfected, *Borrelia*-primed but uninfected and non-primed but *Borrelia*-infected wild-type mice, all of which were non-arthritic.

Figure 2.

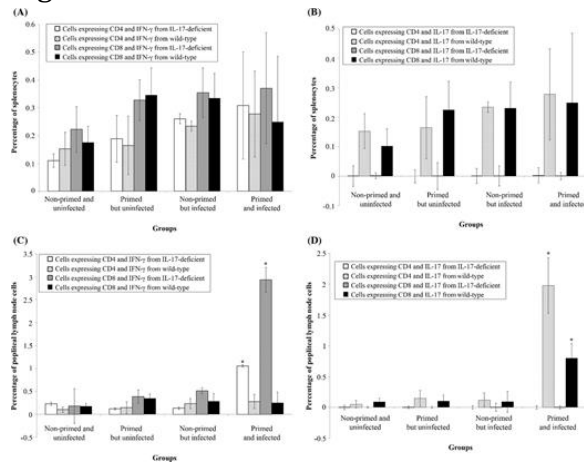


The concentration (picograms per milliliter) of gamma-interferon (A) and IL-17 (B) in sera obtained from IL-17-deficient and wild-type C57BL/6 mice that are non-primed and uninfected, *Borrelia*-primed but uninfected, non-primed but *Borrelia*-infected and *Borrelia*-primed and -infected. Error bars

represent the mean picogram concentration in sera  $\pm$  two standard errors. Asterisks denote a significant ( $P \leq 0.05$ ) difference compared to all other mice with or without the same genotype, and with or without same experimental treatment. Data were obtained from three experiments.

Flow cytometry was conducted to determine whether splenocytes and popliteal lymph node cells, specifically CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes, expressed IFN- $\gamma$  and IL-17. The percentage of CD4<sup>+</sup> and CD8<sup>+</sup> splenocytes expressing IFN- $\gamma$  was not statistically different among non-primed and uninfected, *Borrelia*-primed but not infected, non-primed but *Borrelia*-infected and *Borrelia*-primed and -infected IL-17-deficient and wild-type mice (Fig. 3A). IL-17-expressing CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes in the spleen were not detected in IL-17-deficient mice (Fig. 3B). The percentage of IL-17-expressing CD4<sup>+</sup> and CD8<sup>+</sup> splenocytes was also not significantly different among all groups of wild-type mice.

Figure 3.



The percentage of splenocytes expressing IFN- $\gamma$  (A) and IL-17 (B), and popliteal lymph node cells expressing IFN- $\gamma$  (C) and IL-17 (D) from IL-17-deficient and wild-type C57BL/6 mice that are non-primed and uninfected, *Borrelia*-primed but uninfected, non-primed but *Borrelia*-infected and *Borrelia*-primed and -infected. Asterisks denote a significant ( $P \leq 0.05$ ) difference in percentage of cells compared to all other mice with or without the same genotype, and with or without same experimental treatment. Error bars represent the mean percentage  $\pm$  two standard errors. Data were obtained from three experiments.

However, a significant percentage of IFN- $\gamma$ -expressing CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes was detected among popliteal lymph node cells of *Borrelia*-primed and -infected IL-17-deficient mice and other groups of IL-17-deficient mice compared to *Borrelia*-primed and -infected wild-type mice (Fig. 3C). The percentage of IFN- $\gamma$ -expressing CD4<sup>+</sup> and CD8<sup>+</sup> popliteal lymph node cells in *Borrelia*-primed and -infected wild-type mice was similar to non-primed and uninfected, *Borrelia*-primed but uninfected and non-primed but *Borrelia*-infected IL-17-deficient and wild-type mice. In addition, a significant percentage of IL-17-expressing CD4<sup>+</sup> and CD8<sup>+</sup> popliteal lymph node cells was detected in *Borrelia*-primed and

-infected wild-type mice (Fig. 3D) compared to other groups of wild-type mice. No IL-17 expression was detected among popliteal lymph node cells from IL-17-deficient mice.

## Discussion

IL-17 has been implicated as a contributor to Lyme arthritis (Infante-Duarte *et al.*2000; Knauer *et al.*2007; Codolo *et al.*2008, 2013; D'Elia, Codolo and de Bernard 2009; Oosting *et al.*2011). We previously showed that when *Borrelia*-primed C57BL/6 wild-type mice are infected with *Borrelia burgdorferi*, arthritis develops in the tibiotarsal joints (Nardelli *et al.*2006, 2008; Kotloski *et al.*2008; Kuo *et al.*2011). The arthritis can be destructive, reducing the mobility of *Borrelia*-primed and -infected hamsters (Lim *et al.*1995a,b; Croke *et al.*2000) or IFN- $\gamma$ -deficient mice (Burchill *et al.*2003; Christopherson *et al.*2003; Nardelli *et al.*2004, 2005, 2006; Amlong *et al.*2006). Blocking IL-17 *in vivo* ameliorates the pathology of the tibiotarsal joints in *Borrelia*-primed and -infected wild-type (Nardelli *et al.*2008) and IFN- $\gamma$ -deficient (Burchill *et al.*2003; Nardelli *et al.*2004) mice. In support of the arthritogenic ability of IL-17, Infante-Duarte *et al.* (2000) and others (Codolo *et al.*2008, 2013; D'Elia, Codolo and de Bernard 2009) showed that synovial cells of human Lyme arthritis patients produce IL-17. In addition, IL-17 levels are increased in cerebrospinal fluid of patients with Lyme neuroborreliosis (Henningsson *et al.*2011) and patients with prolonged Lyme disease (Oosting *et al.*2011). Collectively, these results suggest that IL-17 plays a major role in the induction of Lyme-related pathology, including arthritis. However, no direct evidence illustrates that IL-17 is absolutely necessary for the induction and progression of Lyme arthritis.

Here, we report that IL-17 is not essential for the development of arthritis. Our results with wild-type C57BL/6 mice in this study are consistent with our previous findings (Nardelli *et al.*2006, 2008), showing that wild-type C57BL/6 mice infected with viable spirochetes after priming with heat-inactivated *Borrelia* develop arthritis and respond to infection in the perisynovial tissue with an infiltration of predominantly lymphocytes. Significantly elevated IL-17 levels ( $161 \pm 17.5$  pg/ml) in sera of *Borrelia*-primed and -infected wild-type C57BL/6 mice were also observed compared to the other experimental groups, indicating that IL-17 may contribute to the induction of arthritis. In addition, a significant percentage of IL-17-expressing CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes were detected in popliteal lymph nodes of these mice. Since IL-17-producing CD4<sup>+</sup> and CD8<sup>+</sup> cells have been shown to enhance autoimmune encephalomyelitis (Huber *et al.*2013), psoriatic arthritis (Menon *et al.*2014) and other inflammatory diseases, these IL-17-producing cells may be responsible for the *Borrelia*-induced arthritis seen in wild-type C57BL/6 mice primed and

infected with a heterologous strain. However, contrary to our hypothesis, arthritis is also developed in *Borrelia*-primed IL-17-deficient mice upon infection. Hind paw swelling and infiltration of predominantly lymphocytes were detected in the perisynovial tissue. As expected, no IL-17 was detected in their sera or in cells obtained from the spleen or popliteal lymph nodes. These results suggest that IL-17-producing immune cells are not required for the induction of arthritis in *Borrelia*-primed and -infected mice.

What could contribute to the *Borrelia*-induced arthritis observed in IL-17-deficient and wild-type mice? Cytokines often are influenced by a number of immune modulating agents. Doodes *et al.* (2008) showed elevated levels of IFN- $\gamma$  in supernatants of harvested splenocytes from IL-17-deficient mice with proteoglycan-induced arthritis, suggesting a Th1-mediated response in lack of IL-17. We therefore suspect that *Borrelia*-primed IL-17-deficient and wild-type mice have other functioning immune cells, including IFN- $\gamma$ -producing CD4<sup>+</sup> and CD8<sup>+</sup> cells, that can induce arthritis. We show in this study that when *Borrelia*-primed mice are infected with viable spirochetes in the presence or absence of IL-17, a notable IFN- $\gamma$  response is induced. The level of IFN- $\gamma$  in serum of IL-17-deficient *Borrelia*-primed and -infected C57BL/6 mice ( $134 \pm 15.0$  pg/ml) is elevated compared to the level detected in *Borrelia*-primed and -infected wild-type mice ( $81 \pm 12.0$  pg/ml). IFN- $\gamma$  levels were 60 pg/ml or less in IL-17-deficient and wild-type mice that were non-primed and uninfected, *Borrelia*-primed but uninfected and non-primed but *Borrelia*-infected. In addition, the percentage of IFN- $\gamma$ -expressing CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes was greater in the popliteal lymph nodes that drain the arthritic site of *Borrelia*-primed and -infected IL-17-deficient mice compared to the other groups. No difference in expression of IFN- $\gamma$  among the groups was detected with splenocytes. Failure to detect IFN- $\gamma$  in the splenocytes may be the result of a localized immune response with confined processing of *Borrelia* to the popliteal lymph node cells of the hind paws, the initial site of infection.

Our data suggest a role for IFN- $\gamma$  in the induction of arthritis in wild-type mice and mice deficient in IL-17. IFN- $\gamma$  has long been known as an inflammatory agent in later-stage Lyme arthritis (Yssel *et al.*1991; Dong, Edelstein and Glickstein 1997; Yin *et al.*1997; Gross, Steere and Huber 1998; Strle *et al.*2012). Production of IFN- $\gamma$  from predominately CD4<sup>+</sup> Th1 (Yssel *et al.*1991; Yin *et al.*1997; Gross, Steere and Huber 1998; Strle *et al.*2012) and, to a lesser extent, CD8<sup>+</sup> cytotoxic T cells (Dong, Edelstein and Glickstein 1997) has been associated with increased inflammation and severity of *Borrelia*-induced arthritis (Yssel *et al.*1991; Dong, Edelstein and Glickstein 1997; Yin *et al.*1997; Gross, Steere and Huber 1998; Strle *et al.*2012). In this study, IFN- $\gamma$  levels in sera were significantly increased when comparing arthritic *Borrelia*-primed and -infected IL-17-deficient and wild-type mice to non-primed and uninfected, primed but uninfected and non-primed but infected mice with

no major arthritic symptoms. However, we also showed previously that *Borrelia*-primed IFN- $\gamma$ -deficient mice develop severe arthritis after infection with *B. burgdorferi* (Burchill *et al.*2003; Christopherson *et al.*2003; Nardelli *et al.*2004; Amlong *et al.*2006). The arthritis observed in these IFN- $\gamma$ -deficient mice was dependent on IL-17. In support, treatment of *Borrelia*-primed and -infected IFN- $\gamma$ -deficient mice with antibodies directly targeting IL-17 abrogated the development of arthritis (Burchill *et al.*2003; Nardelli *et al.*2004). It is plausible that *Borrelia*-primed mice depleted of IFN- $\gamma$  compensate with an IL-17-mediated inflammatory response to *B. burgdorferi*. With this same reasoning, *Borrelia*-primed and -infected IL-17-deficient mice may promote pro-inflammatory Th1 and IFN- $\gamma$  responses upon infection. In mice with proteoglycan-induced arthritis, overproduction of IFN- $\gamma$  compensates for the absence of IL-17 (Doodes *et al.*2008) and vice versa (Doodes *et al.*2010). Shifting production to IL-17 or IFN- $\gamma$  in these respective animal models of cytokine deficiency could account for the development of arthritis.

In this study, we show that mild histopathological changes occur in the hind paws of IL-17-deficient *Borrelia*-infected mice in the absence of priming. We also show that minimal histopathological changes develop in non-primed but *Borrelia*-infected wild-type C57BL/6 mice, which is consistent with our previous findings (Kotloski *et al.*2008). These two groups respond to infection with a predominant infiltration of neutrophils and mild to moderate infiltration of lymphocytes, respectively in the ankle joint. In contrast, IL-17-deficient or wild-type mice infected with viable spirochetes after priming with heat-inactivated *Borrelia* respond to infection by infiltration of predominantly lymphocytes. The difference in infiltration of predominantly neutrophils or lymphocytes may explain the disparity in the severity of arthritis that develops between *Borrelia*-primed and non-primed IL-17-deficient and wild-type mice. We speculate that borrelial proteins such as neutrophil-activating protein A (Codolo *et al.*2008, 2013) stimulate the innate immune system for influx of neutrophils in non-primed but *Borrelia*-infected IL-17-deficient and wild-type mice. The infection may be better controlled, and development of arthritis is less severe. In contrast, other borrelial antigens encountered during infection may stimulate the acquired immune response induced by *Borrelia* priming with an influx of lymphocytes that augment the severity of arthritis. In support of this hypothesis, CD4<sup>+</sup> T lymphocytes have been shown to be responsible for development of arthritis in *Borrelia*-primed and -infected hamsters (Lim *et al.*1995a,b). This likely explains why the *Borrelia*-priming and -infection model exhibits arthritis in an otherwise arthritis-resistant murine strain. The involvement of T cells is shown in this study by the increased Th1 response in the serum and tissues of these mice, which is exacerbated in the absence of IL-17. However, both the innate and acquired immune responses are involved in response to infection with *B. burgdorferi*. How

the innate and acquired immune responses collaborate to induce arthritis needs to be studied further to provide a more complete picture of the mechanism of Lyme arthritis.

In conclusion, we show that IL-17-deficient *Borrelia*-primed and -infected mice develop arthritis, contrary to our initial hypothesis. A strong IFN- $\gamma$  response was observed in these mice, supporting the known function of this cytokine in the development of disease. Our current findings, coupled with previous studies showing that murine Lyme arthritis can develop in the absence of IFN- $\gamma$  in multiple animal models (Burchill *et al.*2003; Christopherson *et al.*2003; Nardelli *et al.*2004), further support the concept that a series of complex cytokine interactions are involved in the development of Lyme arthritis. Despite our current findings, evidence suggests that IL-17 likely contributes to the development of Lyme arthritis in some animal models (Burchill *et al.*2003; Nardelli *et al.*2004, 2008, 2010; Kotloski *et al.*2008; Kuo *et al.*2011; Hansen *et al.*2013) and may contribute to disease in some human patients (Infante-Duarte *et al.*2000; Knauer *et al.*2007; Codolo *et al.*2008, 2013; D'Elios, Codolo and de Bernard 2009; Oosting *et al.*2011). Further examination of the role IL-17 and IFN- $\gamma$  play in this complex immune response may yield novel therapeutic strategies to treat Lyme arthritis.

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